

This Page Is Inserted by IFW Operations
and is not a part of the Official Record

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

As rescanning documents *will not* correct images,
please do not report the images to the
Image Problem Mailbox.

Active Immunization of Human Ovarian Cancer Patients Against a Common Carcinoma (Thomsen-Friedenreich) Determinant Using a Synthetic Carbohydrate Antigen

*†G. D. MacLean, *M. B. Bowen-Yacyshyn, ‡J. Samuel, *A. Meikle, ¶G. Stuart, ¶J. Nation, *§S. Poppema, ¶M. Jerry, ¶R. Koganty, ¶T. Wong, and *‡¶B. M. Longenecker

*Cross Cancer Institute and Departments of †Medicine, ‡Immunology, and §Pathology, University of Alberta, Edmonton; ¶Tom Baker Cancer Center, Calgary, and ¶Biomira, Inc., Edmonton, Alberta, Canada

Summary: In a phase I study, ten ovarian cancer patients with extensive metastatic disease despite chemotherapy were immunized three to eight times subcutaneously with the synthetic form of the immunodominant disaccharide (β Gal1 \rightarrow 3 α GalNAc) of the Thomsen-Friedenreich antigen conjugated to KLH (TF α -KLH) plus DETOX adjuvant. Six patients were given a "low" dose of TF α -KLH (100 μ g/injection) and four patients were given a "high" dose (500 μ g/injection). All patients received a single low-dose cyclophosphamide treatment (200 mg/m² i.v.) 3 days prior to commencement of the series of immunizations. Immunizations were 2 weeks apart. Little or no toxicity was noted. As expected, all patients (prior to immunization) had naturally occurring IgM antibodies against the synthetic TF α hapten. None of the patients had detectable pre-existing IgG or IgA antibodies against synthetic TF α hapten. Nine of the ten ovarian cancer patients showed a significant increase in IgM titer above pre-existing levels following immunizations with TF α -KLH plus DETOX adjuvant. These same patients also produced IgG anti-TF α and eight of these also produced IgA anti-TF α , although the IgA responses were weaker. Most of the IgG responses followed the IgM responses by 2-4 weeks. Two patients produced a vigorous IgG response after their first TF α -KLH injection, suggesting a recall response. Both direct ELISAs on various solid-phase synthetic carbohydrate antigens and hapten inhibition experiments confirmed the TF α hapten specificity of the antibodies. IgM and IgG anti-TF α -specific antibodies reacted with natural TF antigen, by ELISA and FACS analysis, although the titers were generally lower than the titers against the immunizing TF α hapten. Increased levels of cytotoxic antibodies against TF-expressing tumor cell targets were detected in eight of the ten patients following immunization. One patient who had no detectable cytotoxic antibodies prior to immunization developed increasingly strong cytotoxic antibodies as a function of the number of immunizations. The low antigen dose patients showed as good or better humoral immune responses than the high antigen dose patients. All four high-dose and four of six low-dose patients developed moderate to strong DTH reactions at the vaccination sites. Our results demonstrate that KLH is an acceptable carrier for carbohydrate haptens in humans and that DETOX is an appropriate nontoxic adjuvant for the generation of high-titer specific anti-carbohydrate responses in human cancer patients. To our knowledge, this is the first demonstration that synthetic carbohydrate antigens can be used to generate an immune response in humans against a clinically relevant cancer-associated antigen. **Key Words:** Cancer vaccine—Synthetic carbohydrate—Active specific immunotherapy—Thomsen-Friedenreich—Human immune response.

Received June 28, 1991; accepted December 17, 1991.
Address correspondence and reprint requests to Dr. B. M. Longenecker at Department of Immunology, University of Alberta, Edmonton, Alberta, Canada T6G 2H7.

The Thomsen-Friedenreich (TF) antigen is strongly expressed on the majority of human carcinomas and is very restricted in its expression on the cell surface of normal adult tissue (1). The TF determinant is cryptic in many normal human epithelial tissues, erythrocytes, and lymphocytes, and can be exposed on the cell surface by removal of sialic acid with neuraminidase treatment (1-4). The TF determinant is expressed on the cancer cell surface on certain mucin-like molecules, and is also found on shed mucins (5,6). TF antigen expression on cancer cells has been associated with cancer aggressiveness, metastasis, and poor prognosis (1,7-13). We have previously described the potential of using synthetic carbohydrate antigens, including TF, for the derivation of monoclonal antibodies for immunoscintigraphy and have suggested that the same synthetic determinants could be used for active specific immunotherapy (ASI) of cancer (14-16).

We have used the TA3-Ha murine mammary adenocarcinoma animal model (13,17,18) to show that active immunization with the synthetic immunodominant disaccharide (β Gal1 \rightarrow 3 α GalNAc) of the TF antigen conjugated to keyhole limpet hemocyanin (KLH) can inhibit tumor growth and prolong survival (19-21). A recent paper by Singhal and co-workers (22) demonstrated a similar prolonged survival in TA3-Ha tumor-bearing mice following immunization against Tn, the precursor to TF α . We chose the TA3-Ha model for study because this well-characterized highly metastatic lethal tumor expresses and secretes a mucin called epiglycanin, which expresses multiple repeating TF and Tn carbohydrate epitopes similar to human cancer-associated mucins (5,6). It appears that epiglycanin can protect TA3-Ha tumor cells from the immune system (13,23-26).

Using the animal model, we recently found (27) that low doses of intravenous epiglycanin [mimicking tumor shedding (26)] are highly immunosuppressive, by stimulating suppressor T-cell activity. This suppressor T-cell activity inhibits the anti-TF immune response and enhances the lethality of TA3-Ha tumors (27). Low-dose cyclophosphamide was found to inhibit the epiglycanin-induced suppressor activity, enabling effective immunotherapy by immunization of tumor-bearing mice with synthetic TF hapten conjugated to KLH emulsified in DETOX adjuvant. Such treatment led to long-term (>120 days) survival of up to 95% of the mice, compared with the death of all control mice at approximately 20 days (20,21). We demonstrated that the

antitumor effect and delayed-type hypersensitivity (DTH) reactions induced by immunization were specific to the immunizing TF α hapten (19-21).

Based on these encouraging results and because of the widespread expression of the TF antigen on the majority of human carcinomas (1), we commenced phase I clinical trials with ovarian cancer patients.

The extended phase I studies were designed to test the effect of subcutaneous injections of TF α -KLH with DETOX adjuvant, following a single intravenous treatment with low-dose cyclophosphamide. The patients were closely monitored for DTH skin reactions to the vaccine as well as possible toxicity of the vaccine. In addition, the patients were tested for specific humoral immune responses using our chemically defined synthetic cancer-associated carbohydrate antigens as well as classical cryptic and cancer-associated TF antigen.

MATERIALS AND METHODS

Clinical Trial Design

The patients in the study were treated at the Cross Cancer Institute in Edmonton or the Tom Baker Cancer Centre in Calgary and had identifiable metastatic ovarian cancer, residual or relapsed after at least platinum-based chemotherapy.

The patients at pretherapy evaluation had their blood tested for lymphocyte phenotype profiling and serum tested for anti-TF α antibodies.

Cyclophosphamide was administered prior to the first immunization. All patients were given cyclophosphamide, 200 mg/ml intravenously, followed 3 days later by the first ASI "vaccine" treatment. The vaccine was injected subcutaneously at two sites (0.5 ml each site) in the upper arms and thighs. All patients received TF α -KLH plus DETOX and were immunized initially four times at 2 week intervals. Six patients were immunized with 100 μ g of TF α -KLH plus DETOX (low dose) and four patients were immunized with 500 μ g of TF α -KLH plus DETOX (high dose).

Two weeks following the fourth treatment, patients were fully re-evaluated clinically and immunologically. Patients who had stable or responding ovarian cancer and evidence of an immune response after four ASI treatments were eligible to receive a further four immunizations at approximately 1 month intervals. Patients who were stable and who had no detectable immune response were

eligible to receive a repeat cyclophosphamide injection followed by a further four immunizations at 2 week intervals.

Antigens and Haptens

All haptens and immunoconjugates were provided by Biomira, Inc. (Edmonton, Alberta, Canada): α DGalNAC-OR = Tn; β DGal(1 \rightarrow 3) α DGalNAC-OR = TF α ; β DGal(1 \rightarrow 3) β GalNAC-OR = TF β ; α DNeuNAc(2 \rightarrow 6) α DGalNAC-OR = sialyl-Tn; β DGal(1 \rightarrow 3)[α DNeuNAc(2 \rightarrow 6)] α DGalNAC-OR = sialyl-TF α . R is a linker arm that can be used for covalent attachment of the hapten to a protein carrier.

Various conjugates were generated by covalent linkage of various haptens to KLH or human serum albumin (HSA) as previously described (19,21).

Vaccine Preparation

TF α -KLH was provided as a sterile, pyrogen-free pharmaceutically acceptable formulation by Biomira, Inc. DETOX (RIBI ImmunoChem Research, Inc., Hamilton, MT, U.S.A.) is a sterile, pyrogen-free preparation (28) and is formulated as a lyophilized oil droplet emulsion containing monophosphoryl lipid A and cell wall skeleton from *Mycobacterium phlei*. Immediately prior to injection, the TF α -KLH was reconstituted with phosphate-buffered saline (PBS) and added to the lyophilized DETOX to give a final volume of approximately 1.0 ml and 100 or 500 μ g of TF α -KLH. The final mixture was administered as one-half volume (\sim 0.5 ml) of each dose injected subcutaneously into each of two sites, alternating each treatment between upper arms (deltoid region) and anterolateral thighs.

Purification of TF Mucin and Epiglycanin

Human tumor-associated TF mucin was purified from LS174T cells (a colon adenocarcinoma cell line), as previously reported (6). Briefly, the culture supernatant was concentrated about 20-fold using a High Output Stirred Cell 2000 (Amicon Corporation, Danvers, MA, U.S.A.) with a membrane having a molecular weight cutoff of 100,000. The concentrated supernatant was fractionated on a gel filtration column (Sephacryl S 500 HR; Pharmacia Canada, Baie d'Urfe, Quebec, Canada). The fractions containing the TF mucin were detected by a sandwich radioimmunoassay (RIA) (6) and were pooled and further purified by affinity chromatog-

raphy on a monoclonal antibody (MAb) 49H.8 affinity column.

Epiglycanin was purified from TA3Ha ascitic fluid by the same procedure. Asialoglycophorin, a well-known source of natural TF antigen, was purchased from Sigma Chemical Company (St. Louis, MO, U.S.A.). Glycophorin is the erythrocyte-derived glycoprotein that carries cryptic TF antigen.

Affinity Purification of Anti-TF α Antibodies

Anti-TF α antibodies were purified from patient serum samples by affinity chromatography using a Synsorb T (synthetic TF α hapten immobilized on an inert matrix; supplied by Chembiomed Ltd., Edmonton, Alberta, Canada) column. The serum sample (0.5 ml) was diluted to the Synsorb T column (bed volume of 10 ml). The column was washed with PBS to remove unabsorbed protein, and then eluted with 0.1 M glycine HCl with 0.15 M NaCl (pH 2.8). The eluted fractions were pooled, neutralized with 1 M Tris base, and dialyzed against PBS.

In order to obtain purified anti-TF α IgG, the above sample was loaded on a Protein A column (Pharmacia, Piscataway, NJ, U.S.A.; bed volume of 5 ml), washed with PBS, eluted with 0.1 M glycine HCl with 0.15 M NaCl (pH 2.8), neutralized, and dialyzed against PBS.

ELISA for Anti-TF Antibodies

Microtiter 96-well plates were coated with various haptens conjugated to HSA or various sources of natural TF glycoproteins. Control wells were coated with HSA only. Coated plates were blocked with 0.8% gelatin. Serial dilutions of patients' sera or purified antibodies were incubated on the antigen-coated plates at room temperature for 1 h, after which the wells were thoroughly washed. Alkaline phosphatase-labeled specific anti-human IgA, IgG, IgM, or IgE (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD, U.S.A.) antibodies were added to appropriate wells and incubated at room temperature for 1 h. Each plate was then thoroughly washed and *p*-nitrophenyl phosphate substrate was added to each well. After 30 min at room temperature, 1 M HCl was added to each well to stop the enzyme reaction and the absorbance was read on an enzyme-linked immunosorbent assay (ELISA) reader. Positive control high-titer patient sera were used on each plate to insure reproducibility of re-

sults among plates and assays. Background optical density (OD) readings on HSA-coated wells were subtracted from readings obtained on TF α -HSA-coated wells. The results of the titration are reported as the reciprocal of the highest serum dilution at which the optical density was greater than 0.12.

Hapten Inhibition of the Anti-TF ELISA

An appropriate dilution of immune serum calculated to give an OD of approximately 1.0 when tested with an ELISA with TF α -HSA or asialoglycophorin on the solid phase was mixed with the appropriate dilution of hapten in microtiter plates. All dilutions were made in PBS, pH 7.4. The hapten-serum mixtures were incubated overnight at 2–6°C and the next morning transferred to ELISA plates containing solid-phase TF α -HSA. The covered ELISA plate was then incubated at room temperature for 1 h and the ELISA was developed as described above.

Neuraminidase Treatment of Cells

Neuraminidase treatment of human lymphocytes or leukemia cells was essentially as previously described (3). Briefly, human lymphocytes were isolated from normal donors either using Ficoll Hypaque or a lymphoprep direct draw tube (Becton Dickinson, Mountain View, CA, U.S.A.), washed, and resuspended in PBS at $2\text{--}10 \times 10^6/\text{ml}$. The human T-cell leukemia cell line, Ichikawa (29), was harvested from cell culture. The cells were thoroughly washed and resuspended in PBS. The stock neuraminidase was made up at 1 unit/ml and frozen in 1 ml aliquots at -20°C . An equal volume of stock neuraminidase was added to the cells so that the final concentration of neuraminidase was 0.5 units/ml. The mixture was then incubated at 37°C for 45 min. After incubation, the cells were washed two times in PBS with 1% bovine serum albumin (BSA) and 0.02 M sodium azide. Lymphocytes that have been neuraminidase-treated are referred to as NE lymphocytes (NE-Ly).

Fluorescent Staining

The cells to be stained [human lymphocytes, Ichikawa cells, or 647V cells (a TF antigen expressing human bladder cancer cell line (30))] were resuspended in PBS with 1% BSA and 0.02 M sodium azide. A total of $5 \times 10^5\text{--}1 \times 10^6$ cells were placed

into a V-bottom 96-well plate (Dynatech Labs, Inc., Chantilly, VA, U.S.A.) and spun down. The appropriate dilution of unlabeled primary antibody or a patient's preimmune or immune sera was then added to the cells. The final volume in the wells was approximately 0.06 ml. The mixture was then incubated on ice for 25 min. After incubation, the cells were washed with PBS with 10% BSA and 0.02 M Na azide and then a fluorescein isothiocyanate (FITC)-labeled secondary antibody, either goat anti-human IgM or IgG (Southern Biotech), was added and the mixture was incubated for 25 min on ice. The cells were washed and analyzed immediately on a FACScan (Becton Dickinson), or fixed with 2% paraformaldehyde (PFA) for analysis at a later time. The TF antigen-specific MAb 49H.8 (3) was used as a control to monitor the expression of the TF antigen on the cell surface and was detected with an FITC-conjugated goat anti-mouse IgM antibody (Southern Biotech).

Hapten Inhibition of Antibody Binding to Cell Surfaces

Hapten inhibition was used to determine if the antibody in the patients' sera was specific for the TF α epitope expressed on cell surfaces. Approximately 5×10^5 cells were added to each well of a 96-well V-bottom plate along with an appropriate dilution of patient serum plus the hapten, either TF α or Tn. The cells, sera, and hapten were incubated together for 1.5 h on ice, and then the cells were thoroughly washed. Next, an appropriate dilution of FITC-labeled secondary antibody was added and incubated for 25 min on ice. FITC-labeled goat anti-human IgM and IgG were used as described above. After the final incubation, the cells were washed and immediately analyzed or fixed with 2% PFA for analysis at a later time.

Complement-Mediated Lysis

Ichikawa cells were maintained in RPMI 1640 supplemented with 10% fetal calf serum, 1% L-glutamine, and 0.1% gentamicin. Two days prior to testing, 1.5×10^6 cells were seeded to 10 ml of media and incubated at 37°C and 5% CO_2 . Cells were then harvested and treated with neuraminidase. After 30 min of incubation with neuraminidase, cells were washed twice with PBS plus 1% BSA. A total of 3×10^6 neuraminidase-treated cells were labeled with 600 μCi of ^{51}Cr for 1 h at 37°C . After labeling, cells were washed four times with

PBS and 1% BSA and adjusted to a concentration of 2×10^5 cells/ml.

Dilutions of test sera were made in PBS and 1% BSA. The assay was performed in U-bottom microtiter plates that had been blocked with 0.8% gelatin in TPBS for 2 h at room temperature. Fifty microliters of diluted sera was mixed with 50 μ l (1×10^4) of labeled Ichikawa target cells. Plates were incubated at 4°C for 1 h and then washed using 100 μ l of PBS and 1% BSA. One hundred microliters of 10% complement in PBS (Lo Tox H Rabbit Complement, Cedarlane Labs. Ltd., Hornby, Ontario, Canada) was added to all wells and incubated for 1 h at 37°C. The reaction was stopped by adding 75 μ l of cold PBS and the plates were centrifuged at 1,000 rpm for 10 min. A fraction of the supernatant was collected and counted in a gamma counter. Control wells containing target cells in PBS alone, complement alone, and Triton-10X (total release) were included in each plate.

The percent specific ^{51}Cr release was calculated as follows:

$$\frac{[\text{cpm (experimental)} - \text{cpm (spontaneous release in complement alone)}]}{[\text{cpm (maximum release)} - \text{cpm (spontaneous release in complement alone)}]} \times 100$$

RESULTS

Skin Reactions of Ovarian Cancer Patients

Little or no toxicity was noted following up to eight subcutaneous immunizations with TF α -KLH plus DETOX. Most of the patients developed a

moderate to strong DTH reaction at the vaccine injection site. A typical DTH reaction was noted that first appeared approximately 12 h after immunization. These reactions peaked at 48 h and subsided by 72–96 h. In several cases, small, flat, subcutaneous granulomas (2–15 mm diameter) subsequently developed at injection sites. These gradually subsided over the next 4–6 weeks.

One patient was concerned by a granuloma with overlying induration of approximately 5 cm and she had it incised by someone external to the study. One patient was withdrawn from the study after she had an acute local allergic reaction after the third immunization at one of the two treatment sites. The reaction resolved quickly following intravenous hydrocortisone and diphenhydramine.

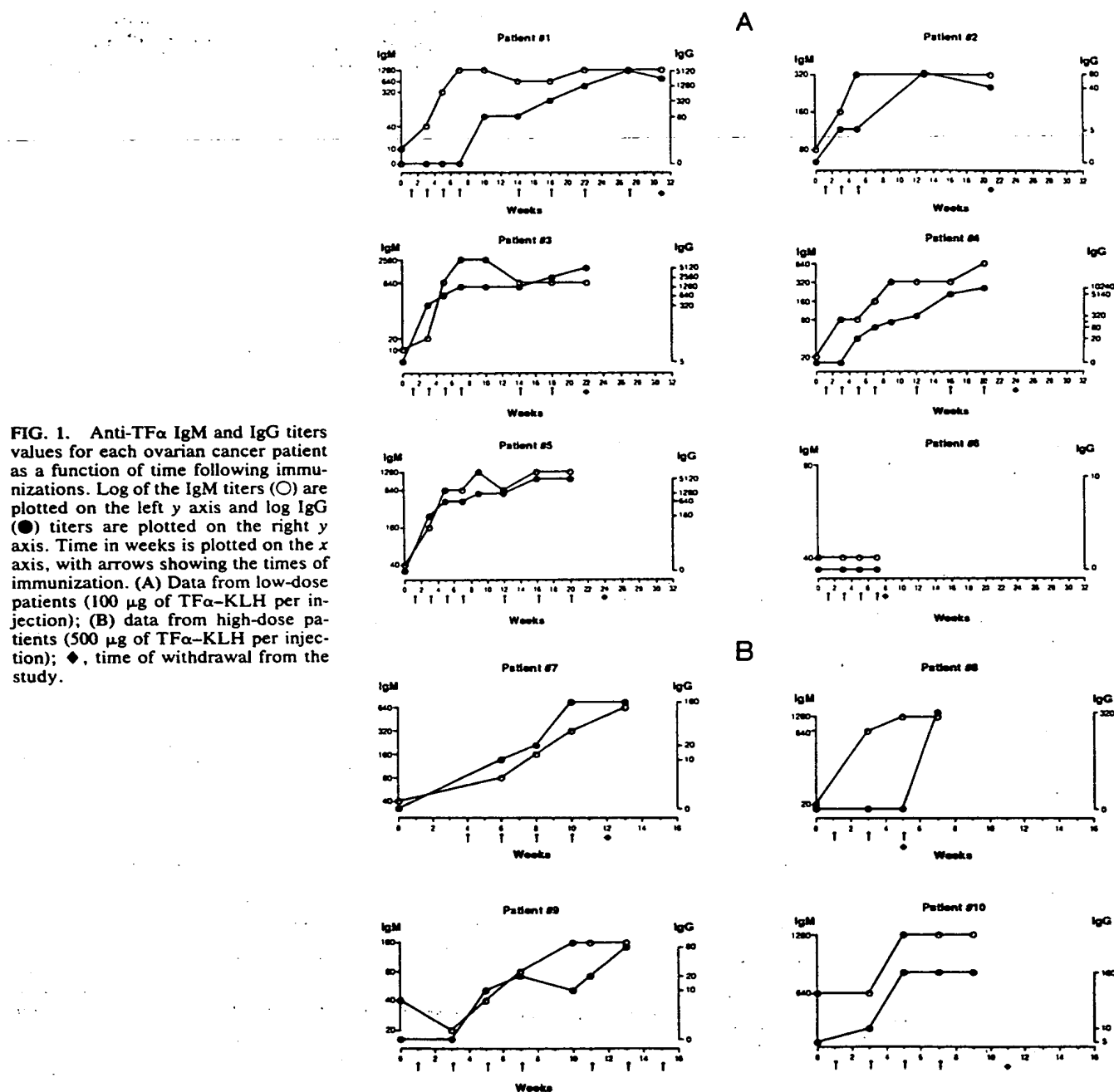
Humoral Immune Response to the Synthetic TF α Hapten

Sera of nine of ten patients demonstrated both an IgM and an IgG anti-TF α hapten response when tested with TF α -HSA using ELISA (Table 1, Fig. 1). Surprisingly, two of the patients (patients 3 and 5) demonstrated a particularly vigorous IgG response following the first immunization that increased in parallel with the IgM response (Fig. 1). The timing and vigor of these responses indicate a memory response to a recall antigen. Eight of ten patients demonstrated an IgA response (Table 1). None of the patients in the study generated detectable IgE anti-TF antibody. One patient (no.6) did not produce any detectable IgM, IgG, or IgA anti-TF antibodies.

TABLE 1. Maximum antibody titers obtained in ovarian cancer patients

Patient no.	No. of immunizations	Test antigen											
		TF α -HSA*						KLH					
		IgM		IgG		IgA		IgM		IgG		IgA	
		Preimmune	Max.	Preimmune	Max.	Preimmune	Max.	Preimmune	Max.	Preimmune	Max.	Preimmune	Max.
Low dose													
1	8	20	1,280	0	5,120	0	20	80	320	40	320	10	320
2	3	80	320	0	80	0	40	160	320	40	320	10	80
3	6	10	2,560	5	5,120	0	160	40	320	40	1,280	10	20
4	7	40	640	0	10,240	0	320	320	640	640	640	80	160
5	7	40	1,280	0	5,120	0	160	160	640	40	1,280	5	40
6	4	40	40	0	0	0	0	320	320	40	160	160	640
High dose													
7	4	40	640	5	160	0	320	320	640	640	640	160	320
8	3	20	1,280	0	320	5	80	80	320	40	320	10	80
9	7	40	160	0	80	0	10	80	640	160	640	10	40
10	4	320	1,280	0	160	0	20	1,280	2,560	160	320	20	40

* Titers are with HSA background OD subtracted.

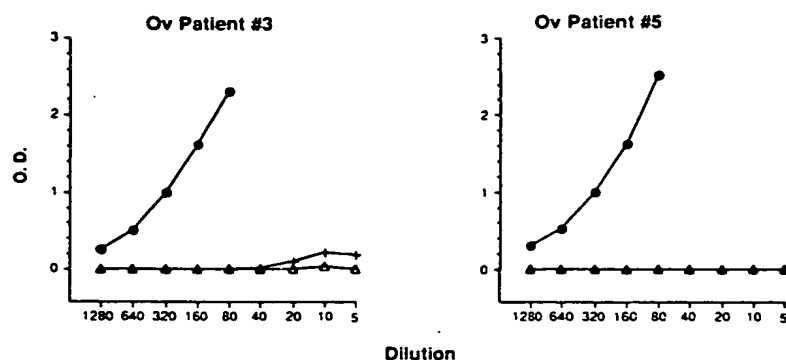


Fine Specificity of IgG Antibodies

The specificity of selected anti-TF α antibodies was determined using two methods: (a) direct binding to various synthetic carbohydrate haptens conjugated to HSA (Fig. 2A) and (b) hapten inhibition

(Fig. 2B). When the IgG antibodies were tested on solid-phase TF α -HSA, sialyl-Tn-HSA, and Tn-HSA, they demonstrated significant binding only to TF α -HSA (Fig. 2A). However, various patterns of cross-reactivity with the different haptens were noted with the various sera in the hapten inhibition

A: SOLID PHASE ANTIGEN



B: HAPTEN INHIBITION

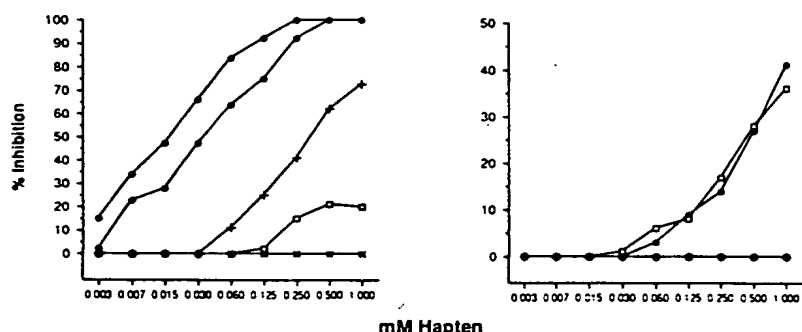


FIG. 2. Tests of IgG antibody specificity. (A) Results of a solid-phase antigen assay (ELISA) performed on a plate coated with TF α -HSA (●), sialyl Tn-HSA (+), or Tn-HSA (Δ), on serum from two patients after two immunizations. (B) Results of inhibition of the direct binding to TF α -HSA on the same sera using five haptens. TF α -crotyl (●), TF β -hexenyl (○), Tn-crotyl (+), sialyl TF α -crotyl (\square), and lactose (\times). Patient no.5 TF β , Tn, and lactose curves are superimposed (all negative inhibitions).

experiments, and the two most predominant patterns are illustrated in Fig. 2B. Thus, the anti-TF α binding of the IgG from patient no.3 was strongly inhibited by both TF α and TF β , mildly inhibited by Tn, weakly inhibited by sialyl-TF α , and not at all by lactose. In contrast, serum from patient no.5 was only inhibited by TF α and sialyl-TF α but not at all by the other three related haptens.

Binding of Anti-TF α Antibodies to Neuraminidase-Treated Cells—A Cell Surface Source of "Cryptic" Human TF Antigen

MAb 49H.8 (3), a specific anti-TF reagent (4,7), has been shown to bind to neuraminidase-treated, but not untreated, human red blood cells (RBCs) and lymphocytes (3,4). Figure 3A shows a FACS scan of anti-TF MAb 49H.8 binding to neuraminidase-treated lymphocytes (NE-Ly) but not untreated lymphocytes, confirming that the neuramin-

idase treatment successfully revealed cryptic TF α hapten. Preimmune sera did not show detectable binding to NE-Ly when tested at a dilution of 1:40, but following immunization, anti-TF IgM (Fig. 3B) and IgG antibodies (Fig. 3D) demonstrated significant binding to NE-Ly when tested at the same dilution. The specificity of the binding was confirmed by using synthetic haptens. TF α hapten but not Tn hapten completely inhibited the binding of both IgM (Fig. 3C) and IgG (Fig. 3D) antibodies to NE-Ly in a dose-dependent fashion (Fig. 4).

Binding of Anti-TF α IgG Antibodies to Purified TF Antigen

Selected immune sera were studied in more detail in order to confirm the specificity of the IgG antibodies for natural TF antigen. Figure 5 demonstrates that increasing titers of antiasialoglycophorin IgG antibodies developed in the latter stages of

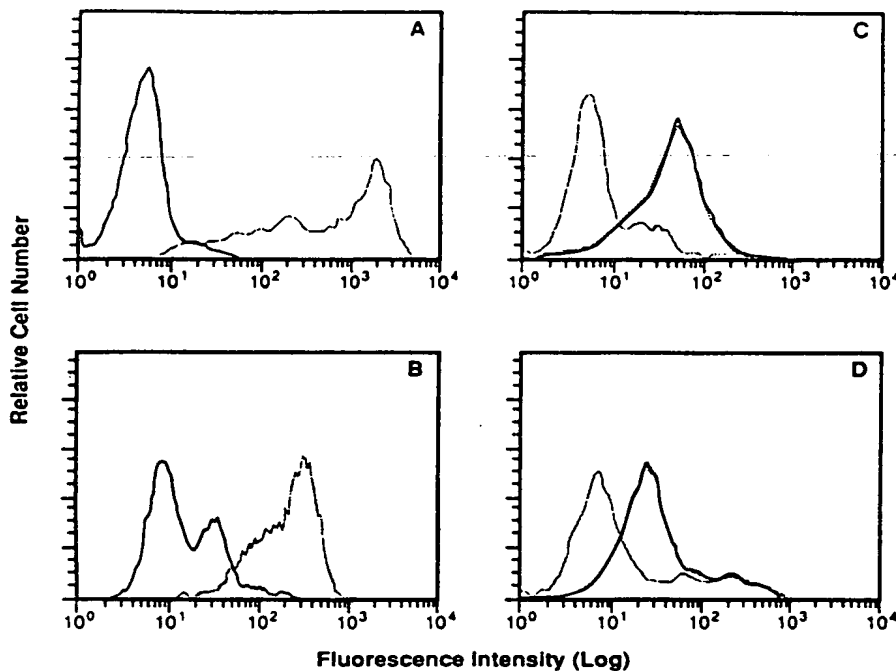


FIG. 3. FACS analysis of binding of anti-TF α serum antibodies to human cell-surface TF antigen. (A) Binding of anti-TF α MA5 49H.8(7) to neuraminidase-treated human lymphocytes (right curve) but not untreated lymphocytes (left curve). (B) Binding of IgM antibodies from immune (right curve) but not preimmune sera (left curve) to neuraminidase-treated human lymphocytes (NE-Ly). Serum dilution tested = 1:40. (C) TF α hapten-specific inhibition of binding of IgM anti-TF antibodies from immune sera to NE-Ly. Left curve represents TF α hapten inhibition while the two right curves are control (no hapten added) or Tn hapten added (negative control). Serum dilution tested = 1:40. (D) TF α hapten-specific inhibition of binding of IgG anti-TF antibodies from immune sera to NE-Ly; same groups as in C. Serum dilution tested = 1:40.

immunization. Human asialoglycophorin is a well-known source of classical human TF antigen (1). Although there was a significant positive correlation between the titers of anti-TF α and anti-asialoglycophorin in various postimmunization sera ($p < 0.001$), the anti-TF α synthetic hapten titers were higher than those measured using asialoglycophorin (compare Figs. 1A and 5). Synthetic TF α hapten but not Tn synthetic hapten totally inhibited the binding of the IgG antibodies to asialoglycophorin, confirming their anti-TF α specificities (Fig. 5).

In order to confirm further the presence of IgG antibodies against human TF antigen, we purified anti-TF IgG by a two-step procedure. In the first step, solid-phase TF synthetic hapten (T-synsorb) was used for the affinity purification of anti-TF antibodies, and in the second phase, the anti-TF antibodies were poured onto a protein A column and the IgG fraction was eluted with 0.1 M glycine HCl with 0.15 M NaCl (pH 2.8). A reducing sodium dodecyl sulfate (SDS) gel revealed the presence of only γ heavy chains plus light chains but not the presence of detectable μ chains (data not shown). Table 2 demonstrates that the purified anti-TF α IgG fraction reacts with not only the hapten used for its purification (TF α), but also three natural sources of TF antigen: epiglycanin, human TF mucin, and asialoglycophorin. Once again, the titers were

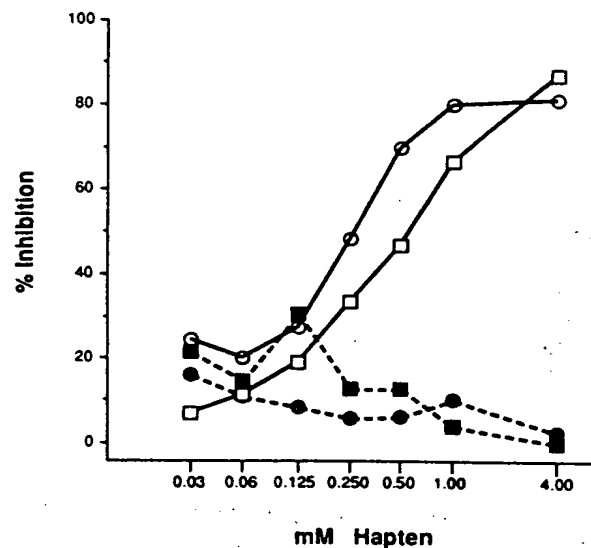


FIG. 4. The presence of specific anti-TF α antibody (IgM and IgG) is shown by hapten inhibition. Immune sera from patient no.3 has specific IgM and IgG anti-TF antibodies that is inhibited from binding to neuraminidase-treated lymphocytes with a range of TF α hapten concentrations. There is little or no inhibition of binding of the IgM and IgG antibodies with the Tn hapten. Serum tested at a 1:40 dilution; ○, IgM test following preincubation of serum with various concentrations of TF α hapten; □, IgG test following incubation with various concentrations of TF α hapten; ●, IgM test following preincubation with various concentrations of Tn hapten; and ■, IgG test following preincubation with various concentrations of Tn hapten.

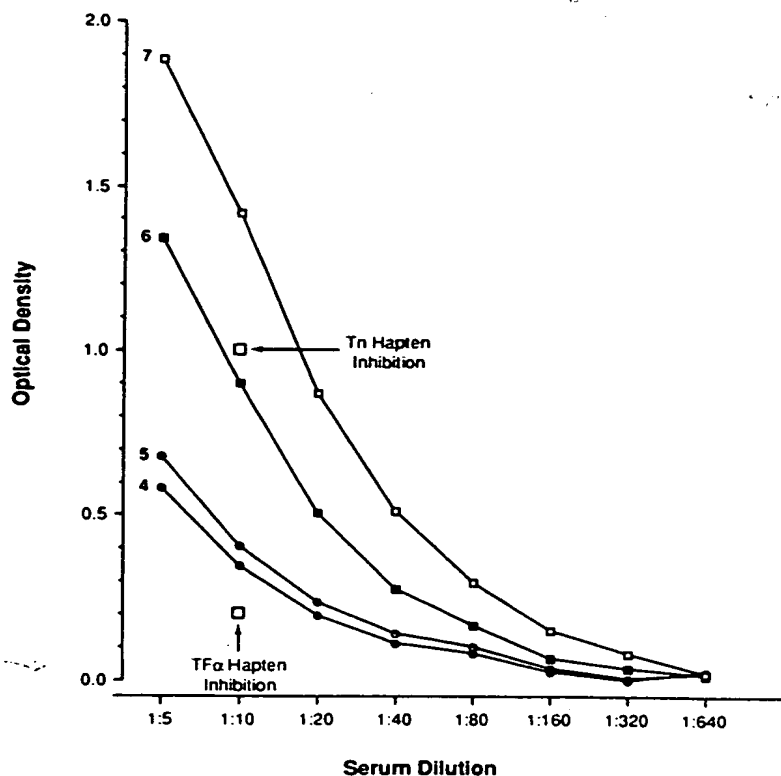


FIG. 5. The development of IgG antibodies against a soluble purified source of human TF antigen. Sera collected following the fourth, fifth, sixth, and seventh immunizations of patient no. 4 were tested at various dilutions on solid-phase asialoglycophorin in an ELISA. The curves generated using the preimmune serum and sera collected following the first, second, and third immunizations are very similar to the fourth immunization curve. □, at 1:10 dilution of serum 7, shows the decreased OD due to inhibition by Tn hapten and TFα hapten as indicated.

higher when tested with synthetic TFα-HSA in comparison with binding to natural sources of TF antigen (Table 2). The purified IgG fraction was also shown to react with 647V human bladder cancer cells (Fig. 6B), a cell line that strongly expresses TF antigen (6,30) as well as a tumor-derived source of cryptic TF antigen, neuraminidase-treated human T-cell leukemia (Ichikawa) cells (Fig. 6A). The binding of the purified IgG fraction to the cells could be specifically inhibited with TFα but not Tn hapten (Fig. 6A and 6B). Finally, the purified IgG fraction and postimmune sera from patient no. 5 was shown to react with an ovarian carcinoma cell line, OVCAR-3, which was found to express the TF antigen detected by Mab 49H.8 (Table 3).

Complement-Mediated Cytotoxicity of Sera from Immunized Patients

In order to determine possible effector function of the anti-TFα antibodies, preimmune sera from all of the patients in the study were compared with selected postimmune sera from the same patients. Eight of the ten patients showed a significantly increased cytotoxic antibody response following immunization (Table 4) and only patients 2 and 6 did not generate detectable cytotoxic antibodies. Patient 8 who had no detectable preimmunization cytotoxic antibodies developed increasingly strong cytotoxic activity following each immunization (Table 4). The TFα specificity of the cytotoxic antibody

TABLE 2. Specificity of purified anti-TF IgG from a patient (no. 1) immunized with TFα-KLH plus DETOX

Concentration of purified IgG (μg/well)	Test antigen				
	TFα-HSA	Epiglycanin	Human TF mucin	Glycophorin	Asialoglycophorin
3.90	>3.00 ^a	2.05	>3.00	0.42	>3.00
0.39	>3.00	0.34	0.42	0.06	0.98
0.19	1.34	0.12	0.17	0.02	0.43

^a Optical density following ELISA test; background on FCS blocked wells <0.07 OD.

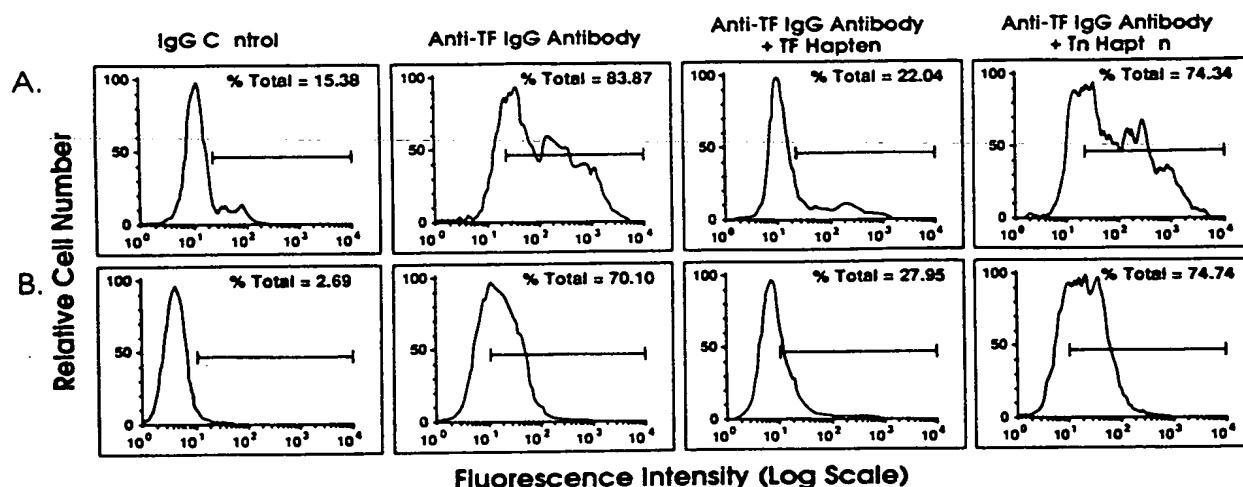


FIG. 6. The binding of purified anti-TF α IgG antibody from patient no.1 to either neuraminidase-treated Ichikawa human leukemia cells (A) or 647V human bladder carcinoma cells (B) was inhibited by the addition of 4 mM TF α hapten, but not 4 mM Tn hapten.

ies was demonstrated by TF α hapten but not Tn hapten inhibition of the cytotoxic activity of the postimmune serum samples (Table 4).

Clinical Observations on Ovarian Cancer Patients

Table 5 summarizes the clinical status and outcome of the ten patients in this study. As this is a nonrandomized pilot study, it would be inappropriate to attempt any conclusions regarding the impact of ASI on their bulky metastatic ovarian cancers. Several patients had disease progression within 2 months, this progression being compatible with their extensive disease. Three patients (nos. 1, 3, and 9) had disease stability for at least 3 months while on therapy. In fact, patient 9 is still stable 4

months after entry into the program but her disease has been intermittently indolent. Patients 1 and 3 had several abdominopelvic masses. One mass in each patient showed a measurable decrease in size early during the study, with subsequent relapse while still in the study.

TABLE 3. Reactivity of purified anti-TF-IgG and postimmune serum IgG with the human ovarian carcinoma cell line, OVCAR-3^a

Antibody	Percent positive cells \pm SD ^b
49H.8 ^c	61 \pm 3
IgM control	7 \pm 2
Purified anti-TF IgG ^d	33 \pm 4
IgG control	9 \pm 3
Preimmune serum ^e	16 \pm 2
Postimmune serum ^f	84 \pm 10

^a Obtained from the American Type Culture Collection.

^b Estimated from FACSCAN analysis.

^c Anti-TF MAb (3).

^d From postimmune serum of patient no. 1, see Table 2.

^e From patient no. 5, sera were diluted 1:20 for test.

^f From serum collected 2 weeks after the seventh immunization.

TABLE 4. Complement-mediated cytotoxicity of preimmunization vs. postimmunization sera

Serum from patient no.	Preimmune	Postimmune ^a	p value
1	18 \pm 9 ^b	42 \pm 7	<0.05 ^c
1 plus TF α hapten	N.D.	21 \pm 7	<0.01 ^d
1 plus Tn hapten	N.D.	40 \pm 3	N.S. ^e
2	16 \pm 7	21 \pm 1	N.S. ^c
3	15 \pm 2	35 \pm 5	<0.02 ^c
4	42 \pm 11	60 \pm 1	<0.01 ^c
5	8 \pm 3	34 \pm 2	<0.001 ^c
6	3 \pm 1	2 \pm 2	N.S. ^c
7	12 \pm 9	32 \pm 6	<0.01 ^c
8	0	28 \pm 9 ^g	<0.001 ^c
	0	44 \pm 6 ^h	<0.001 ^c
	0	55 \pm 9 ⁱ	<0.001 ^c
9	5 \pm 1	15 \pm 4	<0.05 ^c
10	10 \pm 4	22 \pm 1	<0.02 ^c

N.D., not done; N.S., not significant.

^a Serum sample at time of peak anti-TF α IgG titer except for patient no. 8.

^b Percent ⁵¹Cr release from neuraminidase-treated Ichikawa cells \pm SD.

^c Student's *t* test comparing preimmune vs. postimmune sera.

^d Student's *t* test comparing postimmune sera of patient no. 1 with and without haptens (4 mM TF α or Tn hapten).

^e Serum taken 2 weeks after first immunization.

^f Serum taken 2 weeks after second immunization.

^g Serum taken 2 weeks after third immunization.

TABLE 5. *Clinical status of ovarian adenocarcinoma patients*

Patient trial no.	Age (years)	Stage	Grade	Entry date	Extent of disease at entry	Status at completion	Reasons for withdrawal
001	63	III	II	10/31/90	Ascites, large pelvic mass; cystic metastasis in pelvis and upper abdomen	Persisting bulky disease?; progression at 7 months	Study completed
002	47	III	II	10/31/90	Mass above vaginal vault	Bowel obstruction at 1.5 months	Disease progression
003	48	III	III	11/7/90	Two large abdominal masses	Masses enlarging at 3 months	Disease progression
004	68	III	III	12/11/90	Abdominal masses	Progression of palpable disease at 5 months	Patient request
005	51	III	II	12/11/90	Large mass in spleen	Progression at 5 months	Patient request because of concern about granuloma
006	39	III	N/A	12/12/90	Pelvic mass	Bowel obstruction at 1.5 months	Disease progression
007	57	IV	II	1/9/91	Large hepatic metastases, pelvic mass	Enlarging hepatic metastases at 2 months	Disease progression
008	59	III	II	1/30/91	Mass near liver; masses in pelvis	Stable at 1 month; subsequently progressed	Localized acute allergic reactions
009	38	IV	I	2/6/91	Bilateral pleural effusions; ascites, mass in abdomen	Stable on study at 4 months	Still on study
010	58	IV	I	2/28/91	Small pelvic nodule; cystic lesions in liver	Enlarging liver metastasis at 2 months	Disease progression

DISCUSSION

To our knowledge, this study represents the first clinical trial using synthetic cancer-associated carbohydrate antigens in human cancer patients. The clinical results were obtained at two cancer centers using two different doses of TF α -KLH plus DETOX. The clinical program resulted from our successful ASI studies with the TA3-Ha murine mammary adenocarcinoma (19-21), which secretes and sheds a TF-bearing mucin.

Naturally occurring anti-TF IgM antibodies are present in all normal human sera possibly due to antigenic stimulation with cross-reacting carbohydrate antigens on the bacterial flora in the gut (1). The titers of natural anti-TF antibodies have been reported to decrease in carcinoma patients possibly due to the formation of antigen-antibody complexes (1,31). There is a great deal of variation in pre-existing anti-TF IgM titers, especially in cancer patients (1,31). We have confirmed this in the present study as, for example, one patient (no. 10) had a preimmunization titer of 1:320, which exceeded postimmunization titers of two other patients in the study. Immunization caused a significant increase in anti-TF IgM levels in nine of ten cancer patients in our study.

The fine specificity of the anti-TF response was determined using synthetic glycoconjugates in a solid-phase ELISA and synthetic hapten inhibition

of binding of the antibodies to TF α -HSA. Although specific reactivity with TF α hapten was noted in our solid-phase ELISAs, the hapten inhibition experiments revealed varying degrees of cross-reactivity to other related synthetic TF-like haptens. Increased titers of antibodies that reacted with classical cryptic TF antigen were clearly demonstrated in sera from the immunized ovarian cancer patients. This reactivity could be specifically inhibited by the synthetic TF α hapten. The anti-TF α IgG antibodies reactive against purified sources of TF antigen showed lower titers on natural TF than on synthetic TF α hapten, suggesting that the TF disaccharide [β Gal(1 \rightarrow 3) α GalNAc] may be an imperfect mimic of a more complete B-cell epitope. We suggest that in addition to the TF disaccharide, the O-linked serine to which it is attached in natural forms of the TF antigen may generate a more complete B-cell epitope. This hypothesis is currently being tested.

Although preimmune sera did not contain detectable levels of IgG reactive with synthetic TF α hapten, nine of ten patients immunized with TF α -KLH plus DETOX adjuvant produced IgG that reacted specifically with synthetic TF α hapten and some of the patients produced relatively high titers. Most of the patients who developed an IgG response to TF α had a classical delayed response following the initial IgM response, with a gradually increasing IgG titer following sequential immunizations. Of particular

note were two patients treated with the low-dose vaccine who showed an unexpected and vigorous early IgG response following the first immunization. We suggest that they may have been "primed" by previous exposure to a cross-reacting antigen, possibly the natural TF antigen expressed by their cancers.

Eight of the ten patients developed antibody with complement-mediated cytotoxic activity against leukemia cell targets bearing exposed TF antigen. One patient who had no detectable pre-existing cytotoxic antibodies developed a strong cytotoxic response as a function of the number of immunizations. One of the two patients (no. 6) who developed no detectable cytotoxic antibodies was the only patient not to develop any detectable IgM or IgG anti-TF α antibodies detectable by ELISA. The other negative patient (no. 2) developed relatively low IgM and IgG anti-TF α antibodies detectable by ELISA. Interestingly, these two patients were among those whose disease progressed the most rapidly.

Our results suggest that KLH is an appropriate carrier for carbohydrate haptens in humans and that DETOX is an acceptable adjuvant for the generation of high titer specific anticarbohydrate responses in human cancer patients.

As the TF antigen and anti-TF antibodies exist in all patients prior to immunization, the possibility should be addressed that the use of DETOX adjuvant alone might contribute to increased anti-TF antibody levels. To test this possibility, we have analyzed sera from two other clinical trials that employed DETOX as an adjuvant but a different carbohydrate hapten conjugated to KLH. Sera from melanoma patients following immunization with GM2-KLH plus DETOX contained anti-GM2 antibodies but the level of anti-TF antibodies were not elevated above preimmunization levels. In addition, breast cancer patients immunized with sialyl-Tn-KLH plus DETOX produced anti-sialyl-Tn antibodies but not elevated levels of anti-TF antibodies (our unpublished data).

All four high-dose and four of six low-dose patients developed moderate to strong DTH reactions at the vaccination site. The specificity of the DTH reactions elicited at the vaccination sites are unknown but it is likely that the KLH carrier protein was responsible for a major part of the reaction. The early reactions to the vaccine are unlikely due to the presence of the DETOX adjuvant per se (28). However, based on observations in cancer patients

(1) as well as animal model studies (19-21), at least part of the skin reactivity to the vaccine may represent a DTH to TF hapten. Springer has shown that carcinoma patients may have a positive DTH reaction to TF antigen (1). Further work with another series of cancer patients will be required to define the specificities of these DTH reactions. These trials are currently underway.

The feasibility of using ASI as a novel therapy against human cancer has been demonstrated by several investigators using mostly crude extracts of human cancer cells (28,32-34). These encouraging studies are limited by the inability in most cases to measure specific immune responses against defined tumor target antigens. More recent studies have used natural melanoma-associated gangliosides for ASI of human melanoma (35-37) and these have demonstrated the feasibility of using defined cancer-associated carbohydrate antigens for ASI of human cancer. Some of the melanoma studies employed gangliosides adsorbed to bacillus Calmette-Guérin (BCG) or salmonella. Patients who produced the highest antibody titers had the best prognosis (35-38) despite the fact that most responded with only IgM antiganglioside antibodies.

With the precedent (39,40) for generating T-dependent IgG responses to carbohydrate antigens conjugated to carrier proteins, we have employed a similar strategy in the present study with a cancer-associated carbohydrate antigen. In our studies, we used KLH as the carrier for a synthetic carbohydrate hapten and have demonstrated the induction of a strong IgG response to the TF α hapten. We suggest that this approach may be used with other cancer-associated carbohydrate antigens (41) for the development of immunotherapeutic vaccines.

Although many studies demonstrate that antibodies can serve as efficient anticancer molecules against human tumor cells (38,42-47), the actual mechanism(s) of cancer rejection in vivo is still not well understood. "Correlative" studies in animal models appear to emphasize the importance of cell-mediated immune mechanisms (48,49). It may be that several immune mechanisms, including both humoral and cell mediated, act cooperatively (46) in cancer rejection in patients undergoing ASI. One advantage to our approach using defined synthetic cancer-associated antigens is the potential to measure the fine specificities of immune responses to the immunotherapeutic vaccines and to probe their relevance to natural cancer antigens. In the present communication, we emphasize humoral immune re-

sponses, but we are presently developing assays to study the specificities of cell-mediated immune responses against these defined cancer-associated carbohydrate antigens.

The patients have tolerated the ASI very well. In two patients, there appeared to be transient reductions in size of at least one of their measurable masses. However, we will only be able to evaluate the clinical efficacy of ASI in prospective, randomized phase III studies. It is also likely that the most effective ASI with immunotherapeutic vaccines using synthetic antigens will require the use of multi-epitopic vaccines, in view of the heterogeneity of human cancers.

Acknowledgment: This work was supported by a joint University-Industry Award from the Medical Research Council of Canada and Biomira, Inc. We thank A. Berg, B. Christian, M. Hajek, V. Pazderka, L. Proskiwi, T. Tan, and B. Widtman for excellent technical assistance and D. Bodnar, S. Lloyd, and P. Thunberg for excellent assistance in their roles as ASI research nurses.

REFERENCES

- Springer GF. T and Tn, general carcinoma autoantigens. *Science* 1984;224:1198-206.
- Rahman AFR, Longenecker BM. A monoclonal antibody specific for the Thomsen-Friedenreich cryptic T antigen. *J Immunol* 1982;129:2021-4.
- Longenecker BM, Rahman AFR, Leigh JB, et al. Monoclonal antibody against a cryptic carbohydrate antigen of murine and human lymphocytes. I. Antigen expression in non-cryptic or unsubstituted form on certain murine lymphomas, on a spontaneous murine mammary carcinoma, and on several human adenocarcinomas. *Int J Cancer* 1984;33:123-9.
- Wolf MF, Schmitt HR, Schumacher K. Expression of Thomsen-Friedenreich (TF) antigens on lymphocytes. *Cell Immunol* 1989;121:360-5.
- Codington JF, Bhavanandan VP, Bloch KJ, et al. Antibody to epiglycanin and radioimmunoassay to detect epiglycanin-related glycoproteins in body fluids of cancer patients. *J Natl Cancer Inst* 1984;73:1029-38.
- Samuel J, Noujaim AA, MacLean GD, Suresh MR, Longenecker BM. Analysis of human tumor-associated Thomsen-Friedenreich antigen. *Cancer Res* 1990;50:4801-8.
- Wolf MF, Ludwig A, Fritz P, Schumacher K. Increased expression of Thomsen-Friedenreich antigens during tumor progression in breast cancer patients. *Tumor Biol* 1988;9:190-4.
- Ohoka H, Shinomiya H, Yokoyama M, Ochi K, Takeuchi M, Utsumi S. Thomsen-Friedenreich antigen in bladder tumors as detected by specific antibody: a possible marker of recurrence. *Urol Res* 1985;13:47-50.
- Summers JL, Coon JS, Ward RM, Falor WH, Miller AW III, Weinstein RS. Prognosis in carcinoma of the urinary bladder based upon tissue blood group ABH and Thomsen-Friedenreich antigen status and karyotype of the initial tumor. *Cancer Res* 1983;48:934-9.
- Coon JS, Weinstein RS, Summers JL. Blood group precursor T-antigen expression in human urinary bladder carcinoma. *Am J Clin Pathol* 1982;77:692-9.
- Springer GF, Cheingsong-Popov R, Schirmacher V, Desai PR, Tegtmeyer H. Proposed molecular basis of murine tumor cell-hepatocyte interaction. *J Biol Chem* 1983;258:5702-6.
- Dow JA, di Sant'Agneses P, Cockett ATK. Expression of blood group precursor T antigen as a prognostic marker for human bladder cancer treated by *bacillus Calmette-Guerin* and interleukin-2. *J Urol* 1989;142:978-82.
- Codington JF, Frim DM. Cell-surface macromolecular and morphological changes related to allotransplantability in the TA3 tumor. In: Manson LA, ed. *Biomembranes*, Vol. II. New York: Plenum Press, 1983:207-59.
- Longenecker BM, Willans DJ, MacLean GD, Selvaraj S, Suresh MR, Noujaim AA. Monoclonal antibodies and synthetic tumor-associated glycoconjugates to study the expression of Thomsen-Friedenreich (TF)-like and TN-like antigens on human cancers. *J Natl Cancer Inst* 1987;78:489-96.
- MacLean GD, McEwan A, Mackie E, et al. The potential of synthetic tumor-associated glycoconjugates (S-TAGs) for generating monoclonal antibodies for breast cancer imaging and for specific immunotherapy. In: Ceriani R, ed. *Breast cancer immunodiagnosis and immunotherapy*. New York: Plenum Press, 1989:3-11.
- MacLean GD, Longenecker BM. Clinical significance of the Thomsen-Friedenreich antigen. In: Longenecker BM, ed. *Seminars in cancer biology: glycosylation changes associated with malignancy*, Vol 2. Saunders Scientific Publications, 1991:431-40.
- Hauschka TS, Weiss L, Holdridge BA, Cudney TL, Zumpft M, Planinsek JA. Karyotypic and surface features of the murine TA3 carcinoma cells during immunoselection in mice and rats. *J Natl Cancer Inst* 1971;47:343-8.
- Friberg S Jr. Comparison of an immunoresistant and an immunosusceptible ascites subline for murine tumor TA3. I. Transplantability morphology and some physicochemical characteristics. *J Natl Cancer Inst* 1972;48:1463-71.
- Henningson CM, Selvaraj S, MacLean GD, Suresh MR, Noujaim AA, Longenecker BM. T cell recognition of tumor-associated glycoprotein and its synthetic carbohydrate epitopes: Stimulation of anticancer T cell immunity *in vivo*. *Cancer Immunol Immunother* 1987;25:231-41.
- Longenecker BM, Henningson C, MacLean G, Suresh MR, Noujaim AA. Synthetic Thomsen-Friedenreich antigens for the production of anti-carcinoma monoclonal antibodies and generation of anti-carcinoma T cells. In: Reading CL, Hakomori S, Marcus DM, eds. *Altered glycosylation in tumor cells*. UCLA Symposium on Molecular and Cellular Biology, No. 79. New York: Alan R. Liss, Inc., 1988:307-20.
- Fung PYS, Madej M, Koganti R, Longenecker BM. Active specific immunotherapy of a murine mammary adenocarcinoma using a synthetic tumor-associated glycoconjugate. *Cancer Res* 1990;50:4308-14.
- Singhal A, Fohn M, Hakomori S. Induction of α -N-acetylgalactosamine-O-serine/threonine (Tn) antigen-mediated cellular immune response for active immunotherapy in mice. *Cancer Res* 1991;51:1406-11.
- Codington JF, Linsley KB, Jeanloz RW. Immunochemical and chemical investigations of the structure of glycoprotein fragments obtained from epiglycanin, a glycoprotein at the surface of the TA3-Ha cancer cell. *Carbohydrates Res* 1975;40:171-82.
- Friberg S, Lillibook B. Evidence for non-exposed H-2 antigens in immunoresistant murine tumors. *Nature New Biol* 1973;241:112-3.
- Codington JF, Klein G, Cooper AG, Lee N, Brown MC, Jeanloz RW. Further studies on the relationship between large glycoprotein molecules and allotransplantability in the TA3 tumor of the mouse: studies on segregating TA3-Ha hybrids. *J Natl Cancer Inst* 1978;60:811-8.

26. Cooper AG, Codington JF, Brown MC. *In vivo* release of glycoprotein I from the Ha subline of TA3 murine tumor into ascites fluid and serum. *Proc Natl Acad Sci USA* 1974;71:1224-8.
27. Fung PS, Longenecker BM. Specific immunosuppressive activity of epiglycanin, a mucin-like glycoprotein secreted by a murine mammary adenocarcinoma (TA3-Ha). *Cancer Res* 1991;51:1170-6.
28. Mitchell MS, Kan-Mitchell J, Kempf RA, Harel W, Shau H, Lind S. Active specific immunotherapy for melanoma: phase I trial of allogeneic lysates and a novel adjuvant. *Cancer Res* 1988;48:5883-93.
29. Watanabe S, Shimamoto Y, Kameya T, et al. Leukemic distribution of a human acute lymphoblastic leukemia cell line (Ichikawa strain) in nude mice conditioned with whole body irradiation. *Cancer Res* 1978;38:3494-8.
30. Coon JS, Watkins JR, Pauli BU, Weinstein RS. Flow cytometric analysis of heterogeneity in blood groups-related antigen expression in a human urinary bladder carcinoma cell line, 647V. *Cancer Res* 1985;45:3014-21.
31. Bray J, MacLean GD, Dugel FJ, McPherson TA. Decreased levels of circulating lytic anti-T in the serum of patients with metastatic gastrointestinal cancer: a correlation with disease burden. *Clin Exp Immunol* 1982;47:176-82.
32. Hollinshead A, Elias G, Arlen M, Buda B, Mosely M, Scherrer J. Specific active immunotherapy in patients with adenocarcinoma of the colon utilizing tumor-associated antigen (TAA): a phase I clinical trial. *Cancer* 1985;56:480-9.
33. Hoover HC Jr, Surdyke MG, Dangel RB, Peters LC, Hanna MG Jr. Prospectively randomized trial of adjuvant active-specific immunotherapy for human colorectal cancer. *Cancer* 1985;55:1236-43.
34. Euhus DM, Gupta RK, Morton DL. Induction of antibodies to a tumor-associated antigen by immunization with a whole melanoma cell vaccine. *Cancer Immunol Immunother* 1989;29:247-54.
35. Livingston PO, Ritter G, Srivastava P, et al. Characterization of IgG and IgM antibodies induced in melanoma patients by immunization with purified GM2 ganglioside. *Cancer Res* 1989;49:7045-50.
36. Ravindranath MH, Morton DL, Irie RF. An epitope common to gangliosides O-acetyl-GD3 and GD3 recognized by antibodies in melanoma patients after active specific immunotherapy. *Cancer Res* 1989;49:3891-7.
37. Livingston PO, Natoli EJ, Calves MJ, Stockert E, Oettgen HF, Old LJ. Vaccines containing purified GM2 ganglioside elicit GM2 antibodies in melanoma patients. *Proc Natl Acad Sci USA* 1987;84:2911-5.
38. Irie RF, Tai T, Morton DL. Antibodies to tumor-associated gangliosides (GM2 and GD2): potential for suppression of melanoma recurrence. In: Torisu M, Yoshida T, eds. *Basic mechanisms and clinical treatment of tumor metastasis*. Chap. 21. San Diego: Academic Press, 1985:371-84.
39. Lee C. Bacterial capsular polysaccharides—biochemistry, immunity and vaccine. *Immunol* 1987;24:1005-19.
40. Kaghty H, Eskola J, Peltola H, Stout MG, Samuelson JA, Gordon LK. Immunogenicity in infants of a vaccine composed of Haemophilus influenzae type b capsular polysaccharide mixed with DPT or conjugated to diphtheria toxoid. *J Infect Dis* 1987;155:100-6.
41. Hakomori S. Tumor-associated carbohydrate antigens. *Annu Rev Immunol* 1984;2:103-26.
42. Irie RF, Morton DL. Regression of cutaneous metastatic melanoma by intralesional injection with human monoclonal antibody to ganglioside GD2. *Proc Natl Acad Sci USA* 1986;83:8694-8.
43. Yamaguchi K, Furukawa K, Fortunato SR, et al. Human monoclonal antibody with dual GM2/GD2 specificity derived from an immunized melanoma patient. *Proc Natl Acad Sci USA* 1990;87:3333-7.
44. Houghton AN, Mintzer D, Cordon-Cardo C, et al. Mouse monoclonal IgG antibody detecting GD3 ganglioside: a phase I trial in patients with malignant melanoma. *Proc Natl Acad Sci USA* 1985;82:1242-6.
45. Hellstrom I, Garrigues HJ, Garrigues U, Hellstrom KE. Highly tumor reactive, internalizing mouse monoclonal antibodies to Ley-related cell surface antigens. *Cancer Res* 1990;50:2183-90.
46. Primus FJ, Pendurthi TK, Hutzell P, et al. Chimeric B72.3 mouse/human (IgG₁) antibody directs the lysis of tumor cells by lymphokine-activated killer cells. *Cancer Immunol Immunother* 1990;31:349-57.
47. Baker E, Mueller BM, Handgretinger R, Herter M, Yu AL, Reisfeld RA. Effect of a chimeric anti-ganglioside GD2 antibody on cell-mediated lysis of human neuroblastoma cells. *Cancer Res* 1991;51:144-9.
48. Fujiwara H, Takai Y, Sakamoto K, Hamaoka T. The mechanism of tumor growth inhibition by tumor-specific Lyt-1+2- T cells: I. Anti-tumor effect of Lyt-1+2- T cells depends on the existence of adherent cells. *J Immunol* 1985;135:2187-91.
49. Nelson M, Nelson DS. Thy and Ly markers on lymphocytes initiating tumor rejection. *Cell Immunol* 1981;60:34-42.

1944-1945

1946-1947

1948-1949

1950-1951

1952-1953

1954-1955

1956-1957

1958-1959

1960-1961

1962-1963

1964-1965

1966-1967

1968-1969

1970-1971

1972-1973

1974-1975

1976-1977

1978-1979

1980-1981

1982-1983

1984-1985

1986-1987

1988-1989

1990-1991

1992-1993